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EFFECT OF AGRICULTURE PESTICIDES ON ANIMAL FERTILITY

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Principal Investigator:
P. Jorge Chedrese, DVM, MSc. PhD
Professor
Dept. of Obstetrics, Gynecology & Reproductive Sciences.
University of Saskatchewan, 103 Hospital Dr. Saskatoon, SK S7N 0W8
Tel. 966-7739, Fax. 966-8040, Email:chedresj@duke.usask.ca

Collaborators:
Dr. Carmen Agostini, College of Veterinary Medicine, Buenos Aires Province National University, Tandil, Argentina.
Dr. Gloria Perez and Dr. Loro Kojo, Michigan State University, East Lansing, MI, USA.

PROJECT TITLE: Effect of Agricultural Pesticides on Animal Fertility

(a) Project Summary. Canada's agricultural industry has become dependent on the use of pesticides in the forms of insecticides, herbicides, fungicides, and rodenticides. The intense use of these agrochemicals has aided the agricultural industry in controlling crop losses from insects, weeds and diseases. Almost five hundred tons of active ingredients and several times this amount of inert ingredients are used annually in the United States alone. More than 80 percent of all herbicides used in Canada are spread in the Prairie Provinces. In Saskatchewan only, more than 9 million hectares of land are sprayed or dusted for control of weeds and brush, and almost 1 million hectares are treated to control insects and other crop diseases. Because of this intense use, there is an increasing concern that exposure to agrochemicals through food, air and water may adversely affect animal and human health. Several currently used pesticides alter sexual hormone synthesis and/or reproductive function in animals. Therefore, the possibility exists that these compounds can affect the reproductive health of domestic and wild animals in their natural habitats.

The present studies are performed to develop cell culture and animal models and biomarkers to determine how pesticides and environmental pollutants can affect the reproductive/endocrine system of domestic animals and therefore fertility. Pesticides, and other environmental pollutants, have already been found contaminating the ovarian follicular fluid of wildlife, domestic animals and humans. These observations indicate that vulnerable tissue, such as the germinal (oocyte) and endocrine (granulosa) cells of the ovary are directly exposed to chemicals that can adversely affect fertility. Testing the effect of these compounds in large animals *in vivo* is very costly. Therefore, we are developing *in vitro* cell culture and laboratory animal models to test the hypothesis that **"pesticides alters the reproductive/endocrine system and can affect oocyte viability and competence, and early embryo development."** We first engineered porcine stable granulosa cell lines to determine concentrations and combinations of compounds that have the potential to impair fertility. In a second group of experiments, we established a mouse model to study the effects of pesticides on in vivo and in vitro fertilization and on embryo development.

(b) Executive Summary: Animals can be exposed to pesticides present in the environment by ingestion from the water, food and air. Pesticides that affect the endocrine system, called endocrine disrupting substances (EDS), have been found contaminating ovaries and are suspected to impair animal reproduction. Studies involving human and animals in several parts of the world including Canada, revealed that pesticides and/or their metabolites, contaminates animals serum and ovarian follicular fluid. Pesticides present a large source of environmental poisonings in humans, domestic animals and wildlife (Loeffler et al 2001). Organochlorines including DDT, methoxychlor, atrazine, aldrin, dieldrin, endrin, heptachlor, chlordane, lindane, kepone and mirex are known for their chemical stability and lipid solubility. Although DDT has been banned in North America, it is widely used throughout other areas of the world and carried into the atmosphere. Drifting of DDT in the biosphere has resulted in contamination of the terrestrial food chain and aquatic life in the Canadian Arctic (Campagna et al 2001). One of the most notorious examples of pesticide poisoning was observed in Lake Apopka, Florida in which high concentrations of DDT metabolites were detected (Guillette 2000), and associated with sexual organ malformations in alligators (Guillette et al 1994; Vonier et al 1996). Moreover, persistent toxic DDT metabolites have been found to accumulate in adipose tissue, amniotic, and follicular fluid, and are associated with failure to conceive in humans (Jarrell et al 1993; Foster et al 2000; Brim et al 2001; Furusawa, 2002; Younglai et al 2002). Atrazine is a herbicide widely used in North America that is frequently detected in ground and drinking water and soil samples (Barbash et al 2001). Atrazine and the non-estrogenic DDT metabolite, *pp*DDE, have the ability to increase estrogen synthesis by activating the cytochrome P450 aromatase (P450arom) in human endometrial stromal and granulosa cells (Younglai et al 2004; Holloway et al 2005; Foster 2005). These observations support the concept that non-estrogenic EDCs can become indirectly estrogenic by stimulating estrogen synthesis in the ovaries, or affecting other target tissues such as the mammary glands and the endometrium.

Methoxychlor, an analogue of DDT, is a widely used organic pesticide that directly targets the ovary and affects fertility (Cummings and Gray, 1989; Swarts and Eroschenko 1998). Methoxychlor induces ovarian atrophy (Eroschenko et al 1995; Okazaki et al 2001), persistent estrus (Martinez and Swartz 1991; You et al 2003) and follicular atresia (Martinez and Swarts, 1991; Swartz and Corkern 1992). The toxic effects of methoxychlor are specific to the antral follicle affecting granulosa cell steroidogenesis (Crellin et al 2001; Chedrese and Feyles 2001), decreasing the number of healthy antral follicles and increasing atresia (Borgeest et al 2002).

Recently it was demonstrated that the organochlorine insecticide lindane and the organophosphate insecticide dimethoate®, both of which lower serum testosterone levels in animals, block steroid hormone biosynthesis in Leydig testicular cells. These findings raise the possibility that other pollutants may also inhibit steroidogenesis and impair reproduction by targeting expression of the steroidogenic geme. Effects are mediated by reduction in the expression of crucial steroidogenic enzymes, the steroid acute regulatory protein (StAR) and the cytochrome P450 side chain cleavage (P450scc). Expression of the StAR gene is a hormonally-regulated rate limiting step in the transport of cholesterol into the mitochondria

for steroid synthesis, and P450scc is a hormonally regulated rate-limiting steroidogenic enzyme that catalyzes the conversion of cholesterol into pregnenolone.

Other compounds, including the heavy metals present in the environment can also impair reproduction. The heavy metal cadmium (Cd^{2+}), which is found at higher levels in durum wheat, a common crop in the prairies, can produce a wide spectrum of reproductive disorders. Cd^{2+} is a common environmental pollutant much of what is released into the environment can be traced to occupational exposure and pollution from mining, smelting and electroplating, as well as the increasingly intensive use of nickel/cadmium batteries, pigments and plastics (Jarup et al 1998). Its high concentrations in the soil and water supply have made it easily detectable in meat, fish and fruits. Cd^{2+} has no known biological function, and prolonged exposure to it has been linked to toxic effects in humans and animals (Zadorozhnaja et al 2000; Satoh et al 2002). Cd^{2+} is a known inhibitor of ovarian, testicular and placental steroidogenesis and has toxic effects on the primordial germ cells of mammals. Cd^{2+} binds to estrogen receptor(s) and mimics the effect of estrogens in MCF-7 human breast cancer cells and in various other tissues and is a potent carcinogen with paradoxical effects on reproduction. Cd^{2+} can act as an estrogen in the whole animal, inducing conditions ranging from uterine hyperplasia to early onset of puberty (Johnson et al 2003). Even at low concentrations, Cd^{2+} adversely influences estrogen-dependent processes in rodents and is more potent than phytoestrogens, most xenoestrogens and selective estrogen receptor modulators (Safe 2003). The results of our investigations suggest that Cd^{2+} may sensitize the ovary to the effects of EDS, which in turn can affect fertility.

(c) Technical Report

(c.1) In vitro experiments. One of the unique aspects of this project was the use of the porcine stable ovarian granulosa cells, the JC-410 cells (Chedrese et al 1998). These cells were developed in the PI's laboratory and have been characterized for studying the effect of EDCs on steroidogenesis (Rodway et al 1999a & 1999b; Gillio-Meina et al 2000; Swan et al 2002; Chedrese et al 2002; Smida et al 2004). Three groups of cells were engineered to express the promoters of the P450scc and StAR genes.

- The first group was transfected with a plasmid vector carrying 100 bp of the of the P450scc gene promoter (100-P450scc-LUC cells).
- The second group was transfected with a plasmid vector carrying 2320 bp of the P450scc gene promoter (2320-P450scc-LUC cells).
- The third group was transfected with a plasmid vector carrying 1423 bp of the StAR gene (StAR-LUC cells).

All groups of cells were co-transfected with the neomycin resistant gene. Transfections were performed by lipid-mediated incorporation of DNA using lipofectamine®. Cells were selected using the neomycin analogue G418 and tested for specific responses to activator of P450scc and StAR genes transcription, such as the protein kinase-A activators, forskolin and cholera toxin (Figure 1). Cells that responded to these activators were cultured in phenol red-

free media M199 with 5% newborn calf serum. Experiments using these cells were conducted under serum-free conditions.

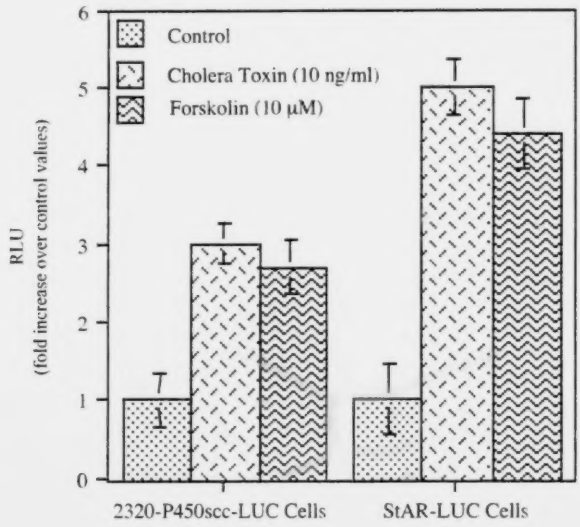

Figure 1. Effect of Protein Kinase-A Activators on Luciferase Activity in the 2320-P450scc-LUC and StAR-LUC cells. Cells were incubated with cholera toxin or forskolin. After 48 h cells were collected and assayed for luciferase activity, expressed as expressed as relative light units (RLU).

(c.1.1) Effect of $CdCl_2$ and Estradiol-17β (estradiol) on Activity of the P450scc Promoter. We tested the hypothesis that $CdCl_2$ interact with estradiol affecting activity of the P450scc gene promoter and cell viability. In a first experimental approach 100-P450scc-LUC cells and 2320-P450scc-LUC cells were exposed to increasing concentrations of estradiol (0.3, 1, 10 & 30 μM) in the presence or absence of $CdCl_2$ (0.6 μM) for 48 hours. Activity of the P450scc gene promoter was assessed by luminometric assay. $CdCl_2$ and estradiol stimulated luciferase activity in the 2320-P450scc-LUC cells, at concentrations between 0.6 & 2 μM (Figure 2A) and 0.3 & 10 μM (Figure 2B), respectively. The effect of estradiol, at all the concentration tested, was potentiated by 0.6 μM $CdCl_2$ (Figure 2B).

The stimulatory effects of E_2 and $CdCl_2$ were not observed in the 100-P450scc-LUC cells (Figure 3). This observation suggest that the estradiol and $CdCl_2$ effect appears to be mediated via a *cis*-acting element located 100 bp upstream of the transcription start site of the P450scc gene promoter (Smida et al., 2004).

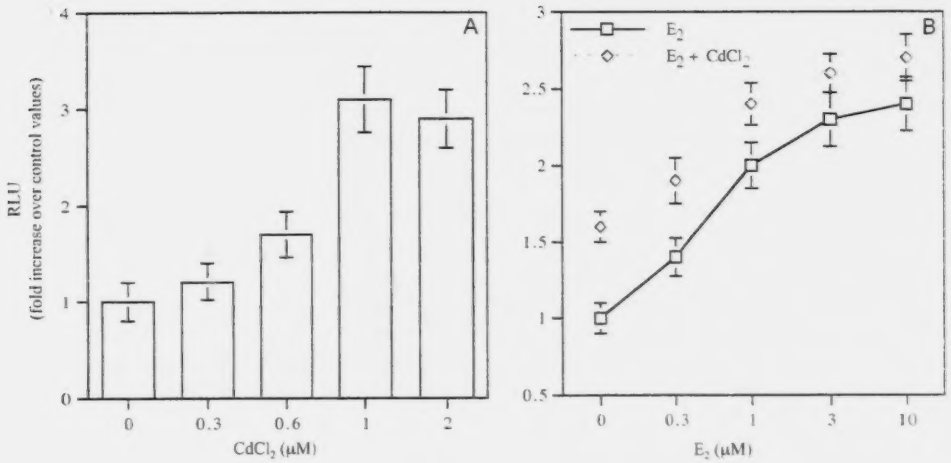

Figure 2. Effect of Estradiol and Cadmium on Activity of the P450scc Promoter. 2320-P450-LUC cells were exposed to increasing concentrations of $CdCl_2$ (A), estradiol (E_2) or E_2+ 0.6 μM $CdCl_2$ (B) After 48 h incubation cells were collected and assayed for luciferase activity, expressed as expressed as relative light units (RLU).

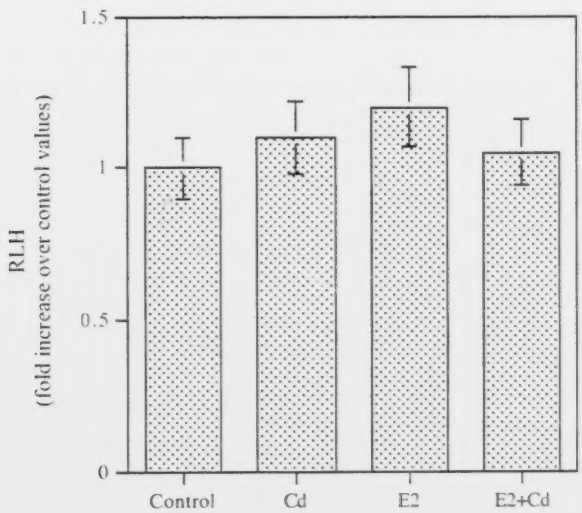

Figure 3. Effect of Cadmium on Luciferase Activity 100-P450-LUC. Cells were exposed to 0.6 mM $CdCl_2$ (Cd), 10 μM estradiol (E_2) or Cd+E_2. After 48 h incubation cells were collected and assayed for luciferase activity, expressed as relative light units (RLU).

(c.1.2) Effect of $CdCl_2$ and ppDDE on Activity of the P450scc Promoter. From the results of our previous studies we know that ppDDE affected expression of the P450scc gene (Crellin et al., 1999; Crellin et al., 2001). Therefore, we tested the combined effects of ppDDE and $CdCl_2$ in the 2320-P450scc-LUC cells. ppDDE increased activity of the P450scc promoter in a dose-dependent fashion, reaching the maximum stimulation of 2.5-fold at 1 ng/ml, after 48 h incubation (Figure 4). A further increase, of 2.6-fold, was observed when the same concentrations of ppDDE were added in the presence of to 0.6 μM $CdCl_2$ (Figure 4).

We conclude that Cd^{2+} stimulates expression of the porcine P450scc gene in the genetically modified stable porcine granulosa cells, JC-410. The effects of Cd^{2+} are potentiated by estradiol and by the DDT metabolite ppDDE. Recent reports suggest that Cd^{2+} may mimic the effects of estradiol by binding to the estrogen receptor (Johnson et al., 2003; Henson & Chedrese, 2004; Chedrese et al., 2006). It has been recently reported that ppDDE can stimulate activity of the P450arom, the enzyme responsible for the synthesis of estradiol in the ovary. Thus, it is reasonable to think that ppDDE may increase estradiol synthesis and, potentiate the effects of Cd^{2+} on the estrogen receptor resulting in transcriptional activation of the P450scc gene in the granulosa cells.

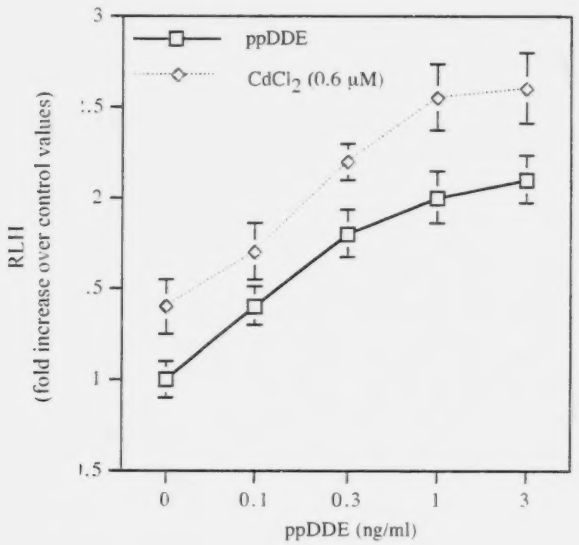

Figure 4. Combined Effects of ppDDE and Cadmium on Activity of the P450scc Promoter. 2320-P450-LUC cells were exposed to increasing concentrations of ppDDE, with and without 0.6 μM $CdCl_2$. After 48 h incubation cells were collected and assayed for luciferase activity, expressed as expressed as relative light units (RLU).

(c.1.3) Effects of $CdCl_2$ and ppDDE on Activity of the StAR Promoter. The studies conducted with StAR-LUC cells showed that the StAR promoter was also stimulated by $CdCl_2$ and ppDDE, but at higher concentrations, 3 μM and 3 ng/ml respectively (Figure 5). In light that these high concentrations also affected cell viability (Figure 6), we did not pursue further studies on the effects of $CdCl_2$ and ppDDE on activity of the StAR Promoter.

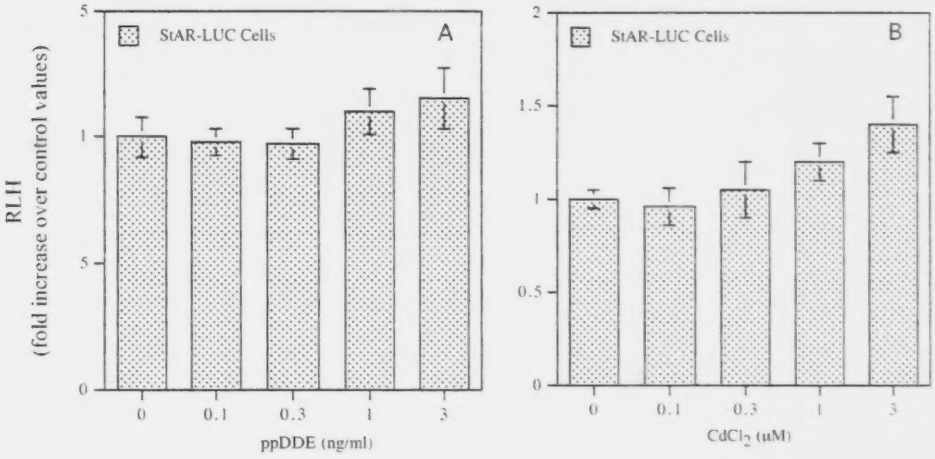

Figure 5. Effects of ppDDE and Cadmium on Activity of the StAR Promoter. StAR-LUC cells were exposed to increasing concentrations of ppDDE or μM $CdCl_2$. After 48 h incubation cells were collected and assayed for luciferase activity, expressed as expressed as relative light units (RLU).

(c.1.4) Effects of Lindane on Activity of the P450scc and StAR Promoters. Recently it was demonstrated that the organochlorine insecticide lindane, which lower serum testosterone levels in animals and block steroid hormone biosynthesis in Leydig testicular cells. These effects appeared to be mediated by an inhibition of the levels of P450scc and StAR. Therefore, we tested the hypothesis that lindane affect transcriptional activity of the P450scc and StAR genes in the 2320-P450scc-LUC and StAR-LUC cells (Figure 7). Lindane inhibited activity of both, the P450scc and StAR promoters, in a dose-related manner (Figure 6). The effect of lindane on 2320-P450scc-LUC was observed starting at 10 μM, while on the StAR-LUC cells was observed starting at 60 μM (Figure 6). Results suggests that lindane is a transcriptional inhibitor of both steroidogenic gene; and that the P450scc promoter is more sensitive than the StAR promoter to the disrupting activity of lindane.

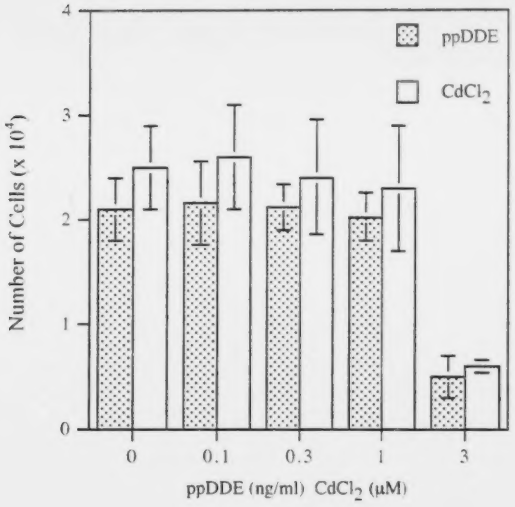

Figure 6. Effects of ppDDE and Cadmium on Cell Viability. StAR-LUC cells were exposed to increasing concentrations of ppDDE or μM $CdCl_2$. After 48 h incubation cultures were washed with buffered saline solution, collected by treatment with trypsine and counted with a hemocytometer.

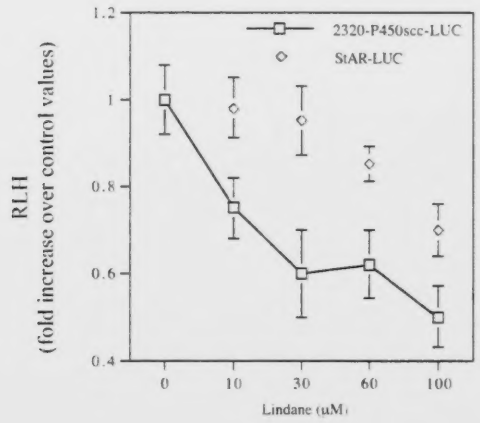

Figure 7. Effect of Lindane on Activity of the P450scc and StAR Promoters. 2320-P450scc-LUC and StAR-LUC cells were exposed to increasing concentrations of Lindane. After 48 h incubation cells were collected and assayed for luciferase activity, expressed as expressed as relative light units (RLU).

(c.1.5) Effects of Cadmium and Estradiol on Granulosa Cells Apoptosis. Exposure of 2320-P450scc-LUC cells to high concentrations of E_2 (30 μM) for 48h induced morphological changes characteristic of apoptosis, including nuclear fragmentation and vacuolation (Figure 8). When the same group of cells were co-incubated with $CdCl_2$ (0.6 μM), fewer apoptotic changes were observed (Figure 8).

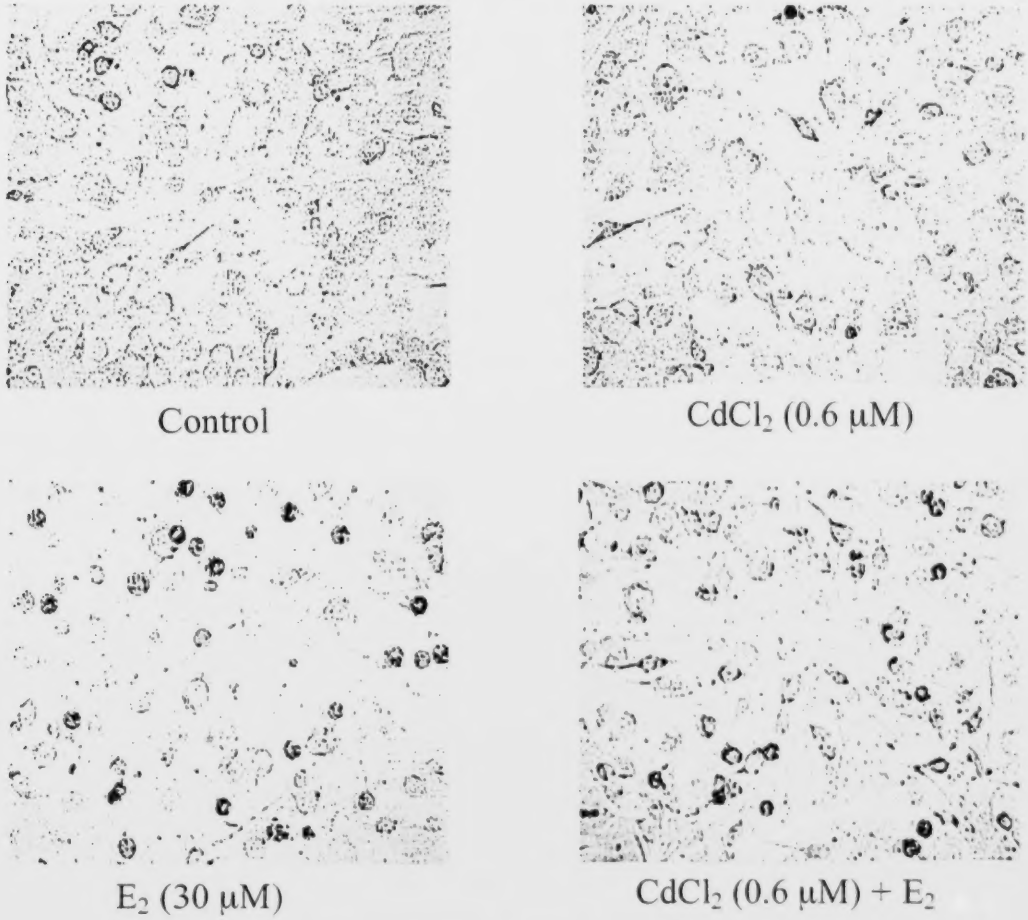

Figure 8. Cells were exposed to 30 μM E_2, in the presence or absence of 0.6 μM $CdCl_2$. Effects on cell morphology were assessed by light microscopy after 48 h incubation and confirmed by epi-fluorescence with the apoptosis-specific stain YOPRO-1.

(c.2) Development of a Mouse Model to Test the Effect of Pesticides on Fertility.

(c.2.1) ***In vitro*** effects of $CdCl_2$ (Cd^{2+}) and Lindane on sperm capacitation in vitro fertilization (IVF) and early embryo development.

Methodology. Superovulation was induced in 6 week old female mice (C57BL/6, B6C3F1, ICR) following a standard protocol (10 IU PMSG/ 10 IU hCG). Cumulus oocyte complexes (COC) were isolated from the oviducts 14 hours after hCG. Intact COCs were added into 25 µl drops and immediately fertilized with 10 µl of sperm. Sperm was either unexposed (CON) or exposed to the chemicals during capacitation (2h) and in some experiments also during IVF. It means sperm was exposed to the chemicals for either 2h, during capacitation only, or 4 h (control sperm in the presence of CC exposed during IVF) or 6 h (2h during capacitation plus 4 h during IVF). A single dose of Lindane or $CdCl_2$ (1 µM) was used.

Results. Cd^{2+} causes a transient decrease in sperm motility, at 4 h of incubation, which is is followed by a full recovery at 6 h. Fertilization rate is not affected, but both 4 h and 2 h incubation with Cd^{2+} affected embryo development.

(c2.2) Effects of Cd^{2+} ***in vivo***

Methodology. A single dose i.p of $CdCl_2$ was given immediately after hCG (from stock solution 1mg/ml dilute 1:10 and inject 200 µl/mouse; an average weight of 20 g), females were mated and next day early in the morning plug (+) females were separated and sacrificed 22 h after hCG.

Results. A single injection of $CdCl_2$ at the time of mating decreases embryo development by 30%.

(c2.3) Effect of Methoxychlor (MTX) and Vinclozolin (VIN) ***in vivo***

Methodology. A single dose i.p of MTX or VIN was given immediately after hCG (from stock solution 1mg/ml dilute 1:10 and inject 200 microliters/mouse; an average weight of 20 g), females were mated and next day early in the morning plug (+) females were separated and sacrificed 22 h after hCG.

Results. A single injection of MTX at the time of mating decreases fertilization rates by 30%. VIN, also injected at the time of mating, caused a complete inhibition of embryo development.

(c.6) Conclusions.

- We developed two engineered cell culture models aimed at investigate the effects of agriculture pesticides and other environmental pollutants, including Cd^{2+}, ppDDE and lindane, on the endocrine/reproductive system of domestic animals.
- Results are in agreement with reports suggesting that Cd^{2+}, a heavy metal with no known physiological function, which pollutes agricultural land and the environment, due to modern industrial processes and waste disposal, can affect animal reproduction by altering gene expression and steroid synthesis in the ovary.
- The observation that Cd^{2+} interact with estradiol supports the concept that the heavy metal binds to the estrogen receptors and has estrogenic properties.
- The interaction between Cd^{2+} and ppDDE supports the concept that a single compound may not always cause an endocrine disruptive effect. Rather, mixture of compounds that are commonly combined in the environment may interact, affecting transcription of the steroidogenic genes required for normal function of the reproductive system and fertility.
- It has been reported that ppDDE stimulates P450arom and estradiol synthesis in the granulosa cells. Therefore, it is reasonable to think that ppDDE indirectly affect P450scc transcription by increasing estradiol synthesis.
- In addition, the observed positive interaction between Cd^{2+} and ppDDE on transcription of the P450scc gene may be explained by an interaction between the estrogenic effect of Cd^{2+} and the effect of ppDDE on intracellular levels of estrogens.
- No effect of Cd^{2+} and ppDDE were observed on the transcriptional activity of the StAR promoter, suggesting that the P450scc may be the main target of the endocrine disrupters that affect ovarian steroidogenesis.
- On the other hand, the pesticide lindane, that also affects steroidogenesis, is able to affect transcription of both genes, the P450scc and StAR.
- Cd^{2+} inhibits apoptosis induced by high concentrations of estradiol. Apoptosis and activity of the P450scc gene promoter are two independent events, since high promoter activity was still observed at concentrations that caused nuclear fragmentation and vacuolation.
- We established a mouse model to study the effects of pesticides on fertility. The information generated suggests that Cd^{2+} causes a transient decrease in sperm motility in the mouse model. Fertilization rate is not affected, however, both 4 h and 2 h incubation with Cd^{2+} affected embryo development. $CdCl_2$ and MTX injected at the time of mating affect embryo development. VIN, also injected at the time of mating, caused a complete inhibition of embryo development.

Overall, results suggest that the models developed are suited to study the mechanisms by which endocrine disrupters affect reproduction. Using these models we also demonstrated that combinations of compounds that are commonly found contaminating the environment could interact and potentiate their disrupting effects in the reproductive/endocrine system.

(d). Personnel. The reported work was conducted by personnel of the PI's laboratory, including Carmen Agostini DVM, PhD and Michael Furlan, whose salaries were paid from

this grant. The in vivo experiments were conducted in collaboration with Dr. Gloria Perez and Dr. Loro Kojo from Michigan State University, East Lansing, MI, USA.

(e). Equipment. No equipment was purchased during this reported period.

(f). Project Development Materials. The following publications were generated from the work conducted using funds granted:

(g). Publications

Perez, G. Kojo, L. & **Chedrese, P. J.** Direct effect of Methoxychlor and Vinclozin Fertilization and Embryo Development in a Mouse Model. *In Progress.*

Chedrese, P. J. Piasek, M. and Henson, M. C. The DDT Metabolite ppDDE Potentiate the Disrupting Effect of Cadmium in Granulosa Cells. *In Progress.*

Piasek, M. Henson, M. C. and **Chedrese, P. J.** Cadmium, a Novel Endocrine/Reproductive Disrupter. Submitted to the 28th International Congress on Occupational Health (centennial ICOH Congress) to be held in Milan, Italy, in June 2006.

Chedrese, P. J. Piasek, M. and Henson, M. C. 2006. Cadmium as an Endocrine Disrupter in the Reproductive System. *Current Medicinal Chemistry- Immunology. In Press.*

Furlan, M., Carver-Ward, J., Clark, R., Urban, R. J. and **Chedrese, P. J**. 2004. Cadmium (Cd^{2+}) Stimulates Transcriptional Activity of the P450scc Gene and Inhibits Apoptosis Induced by Estradiol-17β in cultured JC-410 stable porcine granulosa cells. *Biology of Reproduction Vol. 68, Supp. 1, #83.*

Henson, M. C. and **Chedrese, P. J.** 2004. Endocrine Disruption by Cadmium, a Common Environmental Toxicant with Paradoxical Effects on Reproduction. *Experimental Biology and Medicine, 229:383-392.*

Smida, A., Valderrama, Agostini, C., Furlan, M. and **Chedrese, P. J.** 2004. Cadmium Stimulates Transcription of the Cytochrome P450 Side Chain Cleavage Gene in Genetically Modified Stable Porcine Granulosa Cells, *Biology of Reproduction, 70:25-31.*

(h). Project Photos. See (c3) Results.

(i). Acknowledgment. ADF was acknowledged in the reported publications.

(j). Expense Statement. See attached Financial Report

(k). ICAR Data Entry. N/A.

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